PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Goossens et al.

Serial No.: 10/666,778

Filed: September 18, 2003

For: THE USE OF GENES ENCODING MEMBRANE TRANSPORTER PUMPS TO STIMULATE THE PRODUCTION OF SECONDARY METABOLITES IN BIOLOGICAL CELLS

Confirmation No.: 8721

Examiner: R. Kallis, Ph.D.

Group Art Unit: 1638

Attorney Docket No.: 2676-6085US

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. ALAIN GOOSSENS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dr. Alain Goossens hereby declares:

1. I am a named inventor on the above-referenced patent application.

2. I am a Principle Investigator at the Flanders Institute for Biotechnology, University of Ghent and an expert in the field of plant secondary metabolite biology. A copy of my curriculum vitae is attached.

3. I understand that in the Office Action mailed February 14, 2007, the Examiner has rejected the claims as being obvious over a combination of Theodoulou (Biochem. Biophys. Acta 1465 79-103) (hereinafter "Theodoulou") and Dudler *et al.* (J. Biol. Chem. 267:9 5582-5588)

(hereinafter "Dudler").

4. Attached hereto, I present data showing that expression of a number of genes, other than ABC transporters, known or thought to be involved in secondary metabolite production have no effect on the production levels of four separate alkaloids in the plant cells tested.

5. The experimental data presented herewith indicate that one of ordinary skill in the art would not reasonably expect success in increasing production or secretion of secondary metabolites through the overexpression of genes known or thought to be involved in secondary metabolite synthesis.

6. The experimental data presented herewith further indicate that one of ordinary skill in the art would find an increase in production or secretion of secondary metabolites through the overexpression of ABC transporters to be unexpected.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 14/5/17

Dr. Alain Goossens

Tag[1] cells[3]	Annotation	Accession	pPMT Induction[2]	Alkaloid levels in transgenic			
				NIC	TAB	BAS	TAL
MAP3	AP2 transcription factor	CQ808982	Yes	o	o	o	o
C330	AP2 transcription factor	CQ808845	Yes	o	o	o	o
C476	MAPK kinase	CQ808961	No	o	o	o	o
T172	Protein phosphatase 2C	CQ809147	No	o	o	o	o
MC307	Putative protein	AJ966362	No	NT			
MC410	Putative protein	AJ966363	No	NT			
MC304	Putative protein	AJ966361	No	o	o	o	o
MT101	GTP-binding-Putative protein	CQ809052	No	o	o	o	o
MT401	Glutathione S-transferase	CQ809143	No	+	o	o	o
C17	Putative protein	AJ966358	No	NT			
C18	RNA-binding protein	AJ966359	No	o	o	o	o
C127	GH3-like protein	CQ808735	No	o	-	o	o
C175	GH3-like protein	CQ808768	No	o	o	o	o
MC204	Putative protein	CQ809012	No	o	o	o	o
C406	Putative RNA-binding protein	AJ966360	No	o	o	o	o
C360	Putative protein	CQ808877	No	o	o	o	o
MAP2	Putative protein	CQ808981	No	NT			

[1] Expression of all of these genes is co-regulated with the expression of genes encoding known nicotine biosynthesis enzymes such as PMT and QPRT. List of genes comes from Table 2 of De Sutter et al. (Plant J. 2005).
[2] Capacity of the genes to induce expression of the known nicotine biosynthesis genes was checked in BY-2 protoplasts (De Sutter et al., Plant J. 2005).
[3] Effect of overexpression of the genes on alkaloid accumulation in transgenic BY-2 cells. NIC, nicotine; TAB, anatabine; BAS, anabasine; TAL, anatalline; +, positive effect; -, negative effect; o, no effect; NT, no transgenic cell lines obtained (Häkkinen et al., submitted for publication).

Tag[1] cells[2]	Annotation	Accession	Alkaloid levels in transgenic			
			NIC	TAB	BAS	TAL
C228	Arginine decarboxylase(ADC)	AF321137	o	-	-	-
C308	Ornithine decarboxylase (ODC)	AF233849	-	-	-	-
MC212	Quinolinate phosphoribosyltransferase (QPRT)	AB038494	-	-	-	-

[1] These genes encode enzymes known to catalyze nicotine biosynthesis and their expression is co-regulated with the expression of the other known gene involved in nicotine biosynthesis (PMT).
[2] Effect of overexpression of the genes on alkaloid accumulation in transgenic BY-2 cells. NIC, nicotine; TAB, anatabine; BAS, anabasine; TAL, anatalline; -, negative effect; o, no effect; (Häkkinen et al., submitted for publication).

1) Transformation of BY2 with plasmids pK7WGD2-ScPDR5-US50 and pKWGD2-ScPDR5-W303 increase anatabine production

BY-2 Strain	Nicotine[1]		Anatabine[1]		
	Medium	Cells	Medium	Cells	% in medium
GUS	0	2.00	0.18	157	0.1
ScPDR5-US50	0	0.88	7.40	207	3.6
ScPDR5-W303	0	2.03	5.12	74	6.9

[1] Alkaloid accumulation (indicated in µg/flask, with 20-ml BY-2 culture per flask) in transformed BY-2 cells measured 72 hours after elicitation with 50 µM methyl jasmonate. Results are the mean of three independent experiments.

2) Transformation of Hyoscyamus muticus hairy roots with pK7WGD2-ScPDR5-US50 improved pseudotropin and cuscohygrin production

H. muticus Strain	Tropine[1]	Pseudotropine[1]	Cuscohygrine[1]	Hysocyamine[1]
Control	18	196	56	5008
ScPDR5-US50	12	364	552	1905

[1] Alkaloid accumulation (indicated in µg/g dry weight) in transformed BY-2 hairy roots. Results are the mean of two or more independent transgenic lines.

3) Transformation of Nicotiana tabacum BY2 hairy roots with pK7WGD2-ScPDR5-US50 and pKWGD2-ScPDR5-W303 increased anatabin production and transformation of Nicotiana tabacum BY2 hairy roots with pKWGD2-ScPDR5-W303 increased nornicotine production

BY-2 Strain	Nicotine[1]	Nornicotine[1]	Anabasine[1]	Anatabine[1]	Anatalline[1]
Control	7125	216	956	197	193
ScPDR5-US50	5439	143	161	907	161
ScPDR5-W303	3993	895	150	957	187

[1] Alkaloid accumulation (indicated in µg/g dry weight) in transformed BY-2 hairy roots. Results are the mean of three or more independent transgenic lines.

Short CV Alain Goossens

Personal data

Name: Alain Goossens
Birth date: 18/01/1971
Nationality: Belgian

Academic training & Career

Ghent University, Belgium

- 1988-1990: Bachelor in Sciences, Biology
- 1990-1992: Master in Sciences, Botany-biotechnology
  - *Undergraduate thesis:* *- Title: "Characterisation of mutations occurring during T-DNA transformation and tissue culture of plant cells."*
  - Promoter: Prof. Dr. Marc Van Montagu, Department of Genetics
- 1992-1998: PhD training in Sciences-biotechnology
  - *PhD thesis:* *- Title: "Molecular characterisation of the gene encoding arcelin 5, a seed storage protein from insect resistant wild common beans (Phaseolus vulgaris)."*
  - Promoter: Prof. Dr. Marc Van Montagu, Department of Genetics

Universidad Politecnica de Valencia, Spain

- 1998-2000: Postdoctoral studies
  - Title: "Identification of novel sodium targets and transporters in yeast by characterisation of suppressors of the salt sensitivity of Na^+-ATPase disruptants."
  - Promoter: Prof. Dr. Ramon Serrano, Instituto de Biología Molecular y Celular de Plantas

Flanders Interuniversity Institute for Biotechnology (VIB), Ghent University

- 2000-present: Group leader/Principle Investigator at Department of Plant Systems Biology, with Prof. Dr. Dirk Inzé.
  - Research theme: Plant secondary metabolism and metabolic engineering.

SCIENTIFIC PUBLICATIONS in SCI JOURNALS

1. **Goossens, A.**, Geremia, R., Bauw, G., Van Montagu, M. & Angenon, G. Isolation and characterization of arcelin 5 proteins and cDNAs. *Eur. J. Biochem.* 225: 787-795 (1994).(SCI 3.164)
2. **Goossens, A.**, Ardiles Diaz, W., De Keyser, A., Van Montagu, M. & Angenon, G. Nucleotide sequence of an *arcelin 5-I* genomic clone from wild *Phaseolus vulgaris* (Accession No. Z50202)(PGR95-075). *Plant Physiol.* 109: 722 (1995). (SCI 6.114)

3. Hamelrijck, T.W., Poortmans, F., **Goossens, A.**, Angenon, G., Wyns, L. & Loris R. Crystallographic structure of arcelin-5, a lectin-like defense protein from *Phaseolus vulgaris*. *J. Biol. Chem.* 271: 32796-32802 (1996). (SCI 5.854)
4. Dillen, W., De Clercq, J., **Goossens, A.**, Van Montagu, M. & Angenon G. *Agrobacterium*-mediated transformation of *Phaseolus acutifolius* A. Gray. *Theor. Appl. Genet.* 94: 151-158 (1997). (SCI 3.063)
5. **Goossens, A.**, Dillen, W., De Clercq, J., Van Montagu, M. & Angenon, G. The *arcelin-5* gene of *Phaseolus vulgaris* directs high seed-specific expression in transgenic *Phaseolus acutifolius* and *Arabidopsis* plants. *Plant Physiol.* 120: 1095-1104 (1999). (SCI 6.114)
6. **Goossens, A.**, Van Montagu, M. & Angenon, G. Co-introduction of an antisense gene for an endogenous seed storage protein can increase expression of a transgene in *Arabidopsis thaliana* seeds. *FEBS Lett.* 456: 160-164 (1999). (SCI 3.415)
7. **Goossens, A.**, Quintero, C., Dillen, W., De Rycke, R., Flower Valor, J., De Clercq, J., Van Montagu, M., Cardona, C. & Angenon, G. Analysis of bruchid resistance in the wild common bean accession G02771: no evidence for insecticidal activity of arcelin 5. *J. Exp. Bot.* 51: 1229-1236 (2000). (SCI 3.336)
8. **Goossens, A.**, de La Fuente, N., Forment, J., Serrano, R. & Portillo, F. Regulation of yeast H+-ATPase by protein kinases belonging to a family dedicated to activation of plasma membrane transporters. *Mol. Cell. Biol.* 20: 7654-7661 (2000). (SCI 7.093)
9. **Goossens, A.**, Dever, T.E., Pascual-Ahuir, A. & Serrano, R. The protein kinase Gcn2p mediates sodium toxicity in yeast. *J. Biol. Chem.* 276: 30753-30760 (2001). (SCI 5.854)
10. **Goossens, A.**, Forment, J. & Serrano, R. Involvement of Nst1p/YNL091w and Msl1p, a U2B splicing factor, in *Saccharomyces cerevisiae* salt tolerance. Yeast 19: 193-202 (2002). (SCI 2.301)
11. De Jaeger, G., Scheffer, S., Jacobs, A., Zambre, M., Zobell, O., **Goossens, A.**, De Picker, A. & Angenon, G. Boosting heterologous protein production in transgenic dicotyledonous seeds using *Phaseolus vulgaris* regulatory sequences. *Nature Biotechnol.* 20: 1265-1268 (2002). (SCI 22.738)
12. **Goossens, A.**, Häkkinen, S.T., Laakso, I., Oksman-Caldentey, K.M. & Inzé, D. Secretion of secondary metabolites by PDR-type ABC transporters in plant cell suspension cultures. *Plant Physiol.* 131: 1-4 (2003). (SCI 6.114)
13. **Goossens, A.**, Häkkinen, S.T., Laakso, I., Seppänen-Laakso, T., Biondi, S., De Sutter, V., Lammertyn, F., Nuutila, A.M., Söderlund, H., Zabeau, M., Inzé, D. & Oksman-Caldentey, K.M. A functional genomics approach toward the understanding of secondary metabolism in plant cells. *Proc. Natl. Acad. Sci. USA* 100: 8595-8600 (2003). (SCI 10.231)
14. Zambre, M., **Goossens, A.**, Cardona, C., Van Montagu, M., Terryn, N. & Angenon G. A reproducible genetic transformation system for cultivated *Phaseolus acutifolius* (tepary bean) and its use to assess the role of arcelins in resistance to the Mexican bean weevil. *Theor. Appl. Genet.* 110: 914-924 (2005). (SCI 3.063)

15. Kwade, Z., Swiatek, A., Azmi, A., **Goossens, A.**, Inzé, D., Van Onckelen, H. & Roef, L. Identification of four adenosine kinase isoforms in tobacco by-2 cells and their putative role in the cell cycle regulated cytokinin metabolism. *J. Biol. Chem.* 280: 17512-17519 (2005). (SCI 5.854)
16. Wolucka, B.A., **Goossens, A.** & Inzé, D. Methyl jasmonate stimulates the de novo biosynthesis of vitamin C in plant cell suspensions. *J. Exp. Bot.* 56: 2527-2538 (2005). (SCI 3.336)
17. De Sutter, V., Vanderhaeghen, R., Tilleman, S., Lammertyn, F., Vanhoutte, I., Inzé, D., **Goossens, A.** & Hilson, P. Exploration of jasmonate signaling via automated and standardized transient expression assays in tobacco cells. *Plant J.* 44: 1065-1076 (2005). (SCI 6.969)
18. Rischer, H., Oresic, M., Seppänen-Laakso, T., Katajamaa, M., Lammertyn, F., Ardiles-Diaz, W., Van Montagu, M.C.E., Inzé, D., Oksman-Caldentey, K.-M. & **Goossens, A.** Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. *Proc. Natl. Acad. Sci. USA* 103: 5614-5619 (2006). (SCI 10.231)
19. Van Nieuwerburgh, F.C., Van de Casteele, S.R., Maes, L., **Goossens, A.**, Inzé, D., Van Bocxlaer, J. & Deforce, D.L. Quantitation of artemisinin and its biosynthetic precursors in *Artemisia annua* L. by high performance liquid chromatography-electrospray quadrupole time-of-flight tandem mass spectrometry. *J. Chromatogr. A* 1118: 180-187 (2006). (SCI 3.096)
20. **Goossens, A.** & Rischer, H. (2006). Implementation of functional genomics for gene discovery in alkaloid producing plants. *Phytochemistry Reviews*, in press (2006).